Higher efficiency of the liver phosphorylative system in diabetic Goto-Kakizaki (GK) rats

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Abstract Liver mitochondrial bioenergetics of Goto-Kakizaki (GK) rats (a model of non-insulin dependent diabetes mellitus) reveals a $\Delta\Psi$ upon energization with succinate significantly increased relatively to control animals. The repolarization rate following ADP phosphorylation is also significantly increased in GK mitochondria in parallel with increased ATPase activity. The increase in the repolarization rate and ATPase activity is presumably related to an improved efficiency of F_0F_1-ATPase, either from a better phosphorylative energy coupling or as a consequence of an enlarged number of catalytic units. Titrations with oligomycin indicate that diabetic GK liver mitochondria require excess oligomycin pulses to completely abolish phosphorylation, relative to control mitochondria. Therefore, accepting that the number of operational ATP synthase units is inversely proportional to the amount of added oligomycin, it is concluded that liver mitochondria of diabetic GK rats are provided with extra catalytic units relative to control mitochondria of normal rats. Other tissues (kidney, brain and skeletal muscle) were evaluated for the same bioenergetic parameters, confirming that this feature is exclusive to liver from diabetic GK rats.

Key words: Non-insulin dependent diabetes mellitus; Mitochondrion; ATPase; Oxidative phosphorylation; Goto-Kakizaki rat

1. Introduction

Non-insulin dependent diabetes mellitus (NIDDM) is one of the most common diseases spread over the world and is associated with severe disturbances that spoil the life quality of modern developed societies.

The Goto-Kakizaki (GK) rat has been raised as an animal model of NIDDM. This animal is a spontaneously diabetic rat, produced by selective inbreeding of Wistar rats with the highest glucose values during oral glucose tolerance tests [1]. The GK rat exhibits a defective insulin secretion, and impaired glucose tolerance, but no obesity [2]. Furthermore, it was shown that the impaired glucose induced insulin release in islets from GK rats was associated with a perturbation of mitochondrial oxidative events [3,4], suggesting the occurrence of bioenergetic alterations at mitochondrial level.

Since some of the most important biochemical reactions determining the metabolic disorders observed in diabetes are localized in the mitochondria, the aim of the present study was to evaluate possible alterations at the level of mitochondria bioenergetics in diabetic GK rats as compared to control Wistar rats. In this report, by comparing bioenergetic parameters from different mitochondrial preparations (liver, brain, kidney and skeletal muscle), we clearly show that increased ATPase activity and membrane potential are expressive of an increased efficiency in the oxidative phosphorylation of diabetic GK rats as compared with control animals and that this feature is exclusive to the liver.

2. Materials and methods

2.1. Animals

Male spontaneously diabetic GK rats were from our colony (Laboratory Animal Research Center, University Hospitals, Coimbra). They were kept under controlled light and humidity conditions and had free access to powdered rodent chow (diet C.R.F. 20, Charles Rivers, France) and water. Control animals were normal male Wistar rats of similar age (26 weeks), grown and kept under the same conditions. The life span of GK rats equals that of normal Wistar rats.

2.2. Materials

All reagents and chemicals used were of the highest grade of purity commercially available.

2.3. Preparation of liver mitochondria

Mitochondria were isolated from liver of male Wistar and GK rats (26 weeks) by conventional methods [5], with slight modifications [6].

2.4. Preparation of brain mitochondria

Crude mitochondrial preparations were obtained from brain according to the method described by Dykens [7].

2.5. Preparation of kidney mitochondria

Mitochondria were extracted from a homogenate of rat kidney by differential centrifugation according to Cain and Skilleter [8].

2.6. Preparation of skeletal muscle mitochondria

Rat skeletal muscle mitochondria were prepared according to Bhattacharya et al. [9] with minor modifications.

2.7. Protein determination

Protein was determined by the Bradford method, using bovine serum albumin as a standard [10,11].

2.8. Membrane potential ($\Delta\Psi$) measurements

The mitochondrial transmembrane potential was estimated by calculating transmembrane distribution of TPP^+ (tetraphenylphosphonium) with a TPP^+ selective electrode prepared according to Kamo et al. [12] using a calomel electrode as reference, as previously described [13].

For titration with oligomycin, after addition of succinate mitochondria developed a potential of about −220 mV (negative inside). With the addition of ADP, the potential dropped because ATP synthase

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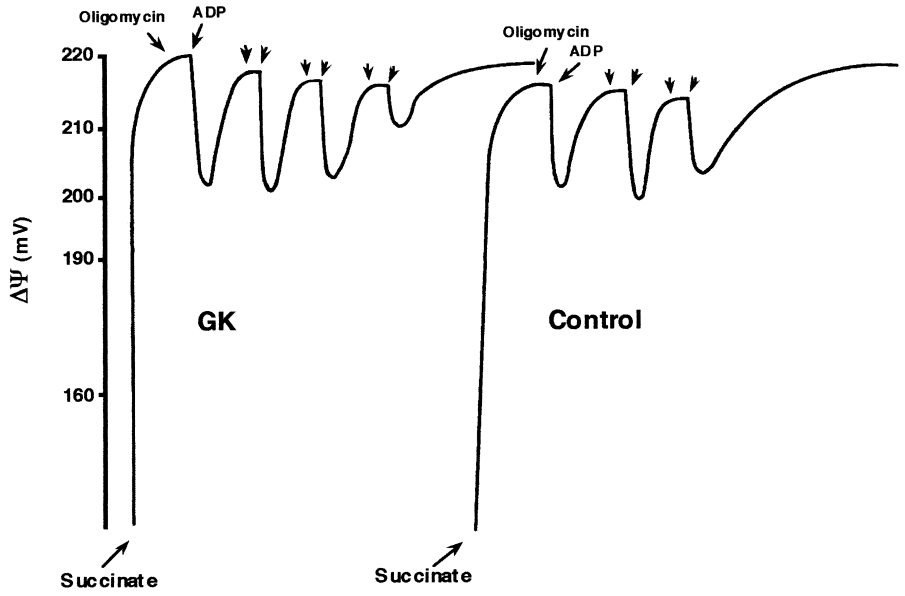

Fig. 1. Titration with oligomycin of mitochondrial membrane potential. Where indicated, oligomycin (0.2 μg/mg protein) and ADP (0.12 mM) were added. The traces represent typical direct recordings from six independent experiments.

uses $\Delta\Psi$ to phosphorylate ADP. After a short lag phase, when ADP phosphorylation takes place, the transmembrane potential repolarized close to the initial value. Successive additions of oligomycin aliquots (0.2 μg/mg protein) 2 min before ADP addition (0.12 mM), as indicated in Fig. 1, progressively block a higher number of ATPase units contributing to the observed decreased number of phosphorylation cycles induced by successive ADP additions.

2.9. ATPase activity

For ATPase activity determination liver, skeletal muscle, kidney and brain preparations were divided into aliquots and frozen in liquid nitrogen for 1–7 days. At the time of use, each aliquot was freeze thawed three times and maintained at 0°C. ATPase activity (liver, skeletal muscle, kidney and brain) was determined by monitoring the pH change in association with ATP hydrolysis, as previously described [14]. The reaction was carried out at 25° C, in 2 ml reaction medium (130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM HEPES, 2 μM rotenone, pH 7.2) and freeze thawed mitochondria (0.5 mg) were added. The reaction was initiated by the addition of 2 mM Mg-ATP; production of H^+ was measured after an elapsed time of 1 min from the start of the reactions, following calibrations of the trace with aliquots of titrated HCl. The addition of oligomycin (1 μg/mg protein) to the medium completely abolished the production of protons. Optimal assay conditions were the same in control Wistar and GK rats.

Brain and kidney mitochondrial ATPase was calculated as the difference in ATPase activity measured in the presence of ouabain (0.2 mM) and of ouabain plus oligomycin (1 μg/ mg protein), as previously described [15].

For kinetic studies, the concentration of ATP ranged from 25 μM to 2 mM. The K_m and V_{max} values were determined from the Lineweaver-Burk analysis.

2.10. Statistics

The results are presented as mean ± S.E.M. of the number of experiments indicated and statistical significance was determined using paired Student's *t*-test.

3. Results

Energization and phosphorylation capacities of mitochondria isolated from diabetic GK and non-diabetic Wistar rats were investigated by following the transmembrane potential ($\Delta\Psi$) developed by mitochondria upon succinate oxidation (Table 1). The results show that the $\Delta\Psi$ values developed in brain, kidney and skeletal muscle from GK rats (Table 1) were similar to those determined in the same tissues from control animals. Additionally, both the depolarization induced by ADP and the repolarization rate showed no difference between GK and control rats (Table 1).

In contrast, $\Delta\Psi$ developed in liver of GK rats upon energization with substrate (Table 1) was significantly increased as compared to controls (from 207.07 ± 2.36 mV in control to 219.17 ± 1.25 mV in GK). Depolarization induced by the ad-

Table 1
$\Delta\Psi$ determined in the presence of succinate as respiratory substrate

Condition	Energization (mV)	ΔADP (mV)	Repolarization rate (% control)
Liver control	207.07 ± 2.36 (n = 11)	17.40 ± 1.10 (n = 11)	100.00 ± 3.94 (n = 9)
Liver GK	219.17 ± 1.25 (n = 9)*	16.08 ± 1.45 (n = 9)	182.34 ± 18.79 (n = 9)**
Brain control	177.9 ± 1.22 (n = 6)	22.8 ± 0.85 (n = 6)	100.0 ± 3.8 (n = 6)
Brain GK	177.4 ± 1.3 (n = 7)	21.8 ± 0.91 (n = 7)	105.2 ± 4.1 (n = 7)
Kidney control	196.5 ± 2.1 (n = 5)	44.8 ± 1.05 (n = 5)	100.0 ± 2.62 (n = 5)
Kidney GK	196.1 ± 2.35 (n = 6)	44.7 ± 1.21 (n = 6)	101.4 ± 3.21 (n = 5)
Smooth muscle control	203.3 ± 2.15 (n = 7)	32.0 ± 0.92 (n = 7)	100.0 ± 3.42 (n = 7)
Smooth muscle GK	202.3 ± 2.42 (n = 6)	33.6 ± 1.25 (n = 6)	98.7 ± 2.81 (n = 6)

Standard reaction medium (1 ml) was supplemented with mitochondria (1 mg) and 3 μM TPP^+ and 2 μM rotenone. The energization of mitochondria was performed by addition of succinate. To induce phosphorylation, 30–40 nmol ADP was used.
Data are mean ± S.E.M. of the number of experiments indicated. Values statistically different from control: *$P < 0.05$; **$P < 0.005$.

Table 2
ATPase activity of diabetic GK and control Wistar rats

Condition	Control	GK
Liver activity (1 min) (nmol H^+/mg/min)	119.54 ± 17.06 (n = 12)	198.61 ± 14.91 (n = 11)**
Brain activity (1 min) (nmol H^+/mg/min)	49.63 ± 6.84 (n = 8)	55.36 ± 8.02 (n = 7)
Kidney activity (1 min) (nmol H^+/mg/min)	88.22 ± 9.55 (n = 8)	98.02 ± 11.31 (n = 8)
Smooth muscle activity (1 min) (nmol H^+/mg/min)	375.35 ± 47.4 (n = 6)	385.54 (n = 6)

Data are mean ± S.E.M. of the number of experiments indicated. Values statistically different from control: $^{**}P < 0.005$.

dition of ADP was similar in liver of GK and controls (17.4 ± 1.10 mV in control and 16.08 ± 1.45 mV in GK), the lag phase preceding repolarization was shortened in the liver of GK rats, but the repolarization rate was highly increased in diabetic liver of GK rats (from 100 ± 3.94% in control to 182 ± 18.79% in GK).

To further explore and understand these findings, the ATPase activity (Table 2) from liver, brain, kidney and skeletal muscle of diabetic GK and control rats was evaluated. As expected, ATPase activities from brain, kidney and skeletal muscle from GK rats (Table 2) were similar to those determined in the same tissues from control animals, with the highest activity obtained in skeletal muscle.

A significantly increased ATPase activity was detected for liver of GK (198.61 ± 14.91 nmol H^+/mg/min) as compared with control animals (119.54 ± 17.06 nmol H^+/mg/min). This increased ATPase activity explains the increased repolarization rate of mitochondria from diabetic liver of GK rat. The improved performance of phosphorylation may be related either to a higher efficiency of F_0F_1-ATPase (increasing the energy coupling) or to a large number of F_0F_1-ATPase units.

Substrate saturation curves for the control Wistar and diabetic GK rats appeared to be almost similar (data not shown). However, when we determined the kinetic parameters for the ATP hydrolysis (Table 3), we observes an identical K_m for both Wistar and GK liver mitochondrial ATPase (87.35 ± 4.3 and 94.5 ± 5.3 μM, respectively in control and GK), suggesting that the affinity for substrate was not altered in liver. In contrast, V_{max} was increased in liver of diabetic GK rats (from 80.3 ± 3.1 to 102.7 ± 5.8 nmol/min/mg, respectively in control and GK), pointing out an increase in specific activity in liver mitochondrial ATPase. Kinetic properties of ATPase determined in brain, kidney and skeletal muscle mitochondria showed no significant differences in K_m and V_{max} values in GK rats as compared with control Wistar rats (data not shown).

The observed differences were further elucidated by titrating the traces with pulses of oligomycin (Fig. 1). It is clear that GK liver mitochondria require a larger number of oligomycin pulses to completely abolish depolarization induced by successive additions of ADP. Therefore, GK liver mitochondria are provided with extra ATPase units as compared with preparations derived from control Wistar rats.

Table 3
Kinetic constants of liver mitochondrial ATPase from control Wistar and diabetic GK rats

Liver	Control	GK
K_m (μM)	87.35 ± 4.3	94.5 ± 5.3
V_{max} (nmol/min/mg protein)	80.3 ± 3.1	102.7 ± 5.8*

Data are mean ± S.E.M. of averages of six independent experiments in each group, derived from the corresponding Lineweaver-Burk analysis. Values statistically different from control: $^{*}P < 0.005$.

4. Discussion

Mitochondria are provided with a variety of bioenergetic functions mandatory for the regulation of intracellular aerobic energy production. Alteration of bioenergetic activities may have drastic consequences on cellular function through the perturbation of energetic charge and balance of the cell.

A review of the literature of experimental diabetes produced by treatment with streptozotocin or alloxan claims attention for defects in mitochondrial function [16–18]. However, at present, little is known about the mechanisms or the progression of these defects. The main difference between the two models (streptozotocin or alloxan induced and GK rats) is that the first model is a drastic and severe model of NIDDM, whereas the GK rat does not present severe complications of the disease, being an appropriate model to study the events at the onset of the disease.

Mitochondrial bioenergetic studies have not yet been carried out in this model (GK rat). Our report for the first time describes an increased efficiency of the phosphorylative system of GK liver mitochondria but not in other tissues like brain, kidney and skeletal muscle.

Since some of the important biochemical reactions determining the metabolic disorders observed in diabetes are localized in the hepatic mitochondria [16] and the liver is a central organ in glucose homeostasis [19], the observed increased efficiency in the phosphorylative system from GK rat liver mitochondria (presumably reflecting a large number of F_0F_1-ATPase units) could develop as a biochemical response or adaptation to circumvent metabolic disturbances associated with NIDDM. The significantly increased kinetic constant (V_{max}) determined for F_0F_1-ATPase from liver mitochondria of GK rats, showing a higher specific activity for the enzyme, supports the hypothesis of increased efficiency of the phosphorylative system in liver mitochondria of GK rats.

A possible explanation could be related to excess use of ATP in some metabolic functions (e.g. pumping of ions or other process) and the improved ATP synthesis would then compensate the defect. In other words, an increased efficiency of oxidative phosphorylation is adequate to maintain an increased turnover of ATP. According to our suggestion, a recent paper from Zong-Chao et al. [20] showed that the utilization of ATP (2.5–3-fold more in the GK than control islets) may justify this increased efficiency on mitochondrial oxidative phosphorylation and suggested that the increased turnover of ATP could be due to altered activity of mitochondrial F_0F_1-ATPase.

Further studies on this subject are being conducted in our laboratory, in order to better elucidate the mechanisms involved in the development of this disease.

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